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14. ABSTRACT

Although surgical resection with adjuvant chemotherapy and/or radiotherapy are used to treat breast tumors, normal tissue tolerance, development of metastases, and inherent tumor resistance to radiation or chemotherapy can hinder a successful outcome. Therefore, it is necessary to consider alternative targeted therapeutic approaches for adjuvant therapy that would significantly reduce undesired side effects in normal tissues. This proposal describes a thermally responsive polypeptide (CPP-ELP-H1) that inhibits breast cancer cell proliferation by blocking the activity of the oncogenic protein c-Myc. The objective of the proposed research is to demonstrate that these genetically engineered polypeptides can be targeted to the tumor site by applying local hyperthermia and can inhibit tumor growth in an animal model. During the first year of this training program, we have used radio-labeled polypeptides to demonstrate that thermal targeting can, in fact, be used to enhance the accumulation of an ELP-based c-Myc inhibitory polypeptide at the tumor site, and we have collected promising tumor reduction data using a lead c-Myc inhibitory polypeptide. Several modifications have been made to the research plan, including altering the way the polypeptide is labeled and adding an additional animal model, and future experiments will complete the proposed aims of determining the optimum CPP for thermal targeting and demonstrating tumor reduction efficacy with the lead CPP-ELP-H1 polypeptide.

15. SUBJECT TERMS
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Introduction

We have developed a thermally responsive polypeptide (CPP-ELP-H1) that inhibits c-Myc transcriptional activity and breast cancer cell proliferation [1]. The objective of the proposed research is to demonstrate that after systemic administration *in vivo*, these genetically engineered polypeptides can be targeted to a breast tumor site by applying local hyperthermia and inhibit tumor growth. The amino acid sequence of the thermally responsive polypeptides is based on the elastin-like (ELP) biopolymer, which is soluble in aqueous solution below physiological temperature (37 °C), but aggregates when the temperature is raised above 41 °C [2]. A cell-penetrating peptide (CPP), bactenecin (Bac), penetratin (Pen), or Tat, is conjugated to the ELP to enhance delivery of the polypeptide across the plasma membrane of the tumor cells. To the CPP-ELP is added a peptide derived from helix 1 (H1) of the helix-loop-helix domain of c-Myc [3], which inhibits transcriptional activation by c-Myc and consequently inhibits cancer cell proliferation [1, 4].

Our hypothesis is that intravenously delivered thermally responsive c-Myc inhibitory polypeptides are likely to be cleared under physiological conditions (37 °C). However, they will accumulate in breast tumors grown in mice, where externally induced local heat (40-42 °C) will be applied. The accumulated polypeptides will block c-Myc activity and consequently inhibit proliferation of the cancer cells. In order to address this hypothesis, the following specific aims were proposed: (1) To quantitate the *in vivo* distribution of CPP-ELP-H1 in normal and neoplastic tissue in an athymic tumor-bearing mouse model of breast cancer and (2) to evaluate the therapeutic efficacy of CPP-ELP-H1 in the treatment of neoplastic xenografts in the mammary tissue of athymic mice with and without localized hyperthermia. Successful completion of the proposed study will provide the first evidence that ELP can deliver a therapeutic molecule and reduce breast tumor size in a thermally targeted manner, and this work will obtain the necessary toxicity, pharmacokinetic, biodistribution, and efficacy data necessary to advance this technology toward the ultimate goal of human therapeutics. Specific targeting of the proposed therapeutic polypeptides to breast tumors by local hyperthermia would increase specificity and efficacy of treatment and reduce the cytotoxicity in normal tissues. Therefore, the proposed research may have a significant impact, leading this technology into clinical trials, and it may provide a powerful technology to treat and manage breast cancer.

Body

Training Program

Learning Animal Techniques. During the first year of this training program, much of my time has been spent learning and practicing experimental techniques in animal models. These techniques include various surgeries, such as tumor implantation and arterial and venous catheter placement; intravenous and intraperitoneal drug administration; histological techniques, including cutting tissue sections with a cryomicrotome and performing various staining techniques (H&E and immunostaining); and data collection and analysis, including quantitative analysis of autoradiograms and fluorescence images. These techniques have been and will be invaluable for my future career as an independent breast cancer researcher. Without these tools, I would not be able to effectively carry out drug development and testing in animal models.

Coursework. As outline in the original proposal, I successfully completed a histology course taught by my Co-mentor Dr. Hamed Benghuzzi. This course taught me the basics of preparing tissue for sectioning and identifying various tissues and cell types within them. Dr. Benghuzzi continues to mentor me to develop my knowledge of histology and to be able to use these techniques to identify signs of toxicity or cellular stress. During completion of Specific Aim 2 of the proposed research, Dr. Benghuzzi will instruct me in analysis of tissue sample from animals treated with the polypeptides we are developing to spot signs of toxicity and hallmarks of side effects that the proposed polypeptides may cause.

Mentorship. I continue to work daily with my Co-mentor, Dr. Drazen Raucher, who oversees all aspects of the drug development and testing. I meet regularly with Dr. Benghuzzi, who, in addition to the hands on instruction described above, also follows my progress on the proposed research with helpful advice. Dr. Benghuzzi has also suggested other animal experiments and applications for the polypeptides under development and offered collaborative help to carry out these experiments. These suggestions include using implantable ceramic controlled release systems to mediate slow release of the therapeutic polypeptides for breast cancer therapy, thereby eliminating the need to perform multiple injections of the agents. In addition to mentorship by Dr. Raucher and Dr. Benghuzzi, I have also enjoyed the benefit of instruction in other animal surgeries by Dr. Eddie Perkins, Assistant Professor of Neurosurgery at UMMC, who taught me to administer glioma cells to develop brain tumors in a rat model of glioblastoma and to monitor these tumors by MRI scanning; and by Dr. David Stec, Assistant Professor of Physiology at UMMC, who taught me to place indwelling venous catheters in the jugular vein of mice and to tunnel the catheters out the back of the animal's neck for repeated administration of compounds via the venous route. I also attended the 2009 LINKS meeting in Washington, DC, which was a unique opportunity to interact with other very bright postdoctoral fellows and with highly distinguished leaders in breast cancer research. I gained an enormous amount of knowledge from this meeting, not only specific to my research, but also about the state of breast cancer research in general and the areas of new development in the field. In addition to the mentorship I have received, I have also served as a mentor to incoming graduate students, teaching them the basics of drug delivery techniques, the hurdles of breast cancer therapy, and the use of animal models to test new approaches for breast cancer treatment.

In vitro Optimization of a Lead Polypeptide Construct

In order to enhance uptake of ELP-based polypeptides, we fused them with cell penetrating peptides (CPPs). CPPs are short peptides known to enhance the cellular uptake of large cargo. The three CPPs proposed for this study are the penetratin (Pen) peptide from Antennapedia [5], the Tat peptide from the HIV-1 Tat protein [6], and the Bac peptide from the bovine antimicrobial bactenecin peptide Bac 7 [7]. We proposed to use these CPPs for delivery of the thermally responsive ELP polypeptide fused to a peptide inhibitor of c-Myc. Previously, we conjugated the c-Myc inhibitory peptide and the Pen peptide to ELP for thermally targeted delivery (Pen-ELP-H1) [1]. Uptake of Pen-ELP-H1 in MCF-7 cells was increased by both the Pen peptide and by the hyperthermia-induced aggregation of ELP. It was demonstrated that Pen-ELP-H1 could sequester endogenous c-Myc to the cytoplasm, thus preventing its interaction with Max or other nuclear partners and its activation of transcription. The result of this inhibition was reduction in the proliferation rate of the MCF-7 cells. In addition, Pen-ELP-H1 increased the potency of topoisomerase II inhibitors, demonstrating potential utility for combination therapy [8]. However, after a short exposure to Pen-ELP-H1, inhibition of cell proliferation was only observed 11 days later. In an attempt to increase the potency of the ELP-H1 polypeptide before beginning *in vivo* testing, it was fused to the Tat or Bac CPPs. We determined the cellular uptake, subcellular distribution, and proliferation inhibition for each construct. The results of this *in vitro* testing were recently published [9], and are included in the attached Appendix 1. To summarize the major findings, all three CPP-ELP-H1 constructs were capable of inhibiting the proliferation of MCF-7 breast cancer cells. Of the three

CPPs, the Pen peptide delivered the most cargo into the cells, but the Bac-ELP-H1 polypeptide was the most potent inhibitor of breast cancer cell proliferation. It was determined that the Bac peptide was capable of delivering the ELP-H1 polypeptide into the nucleus of some of the target cells, whereas Tat and Pen both delivered the cargo polypeptide into the cytoplasm only. We concluded that, although the total cellular uptake was lower for Bac-ELP-H1, its ability to reach the nucleus, and likely interfere with the c-Myc/Max interaction directly, made this construct more potent than the Pen or Tat – containing constructs. Therefore, we elected to use Bac-ELP-H1 as the lead polypeptide for *in vivo* testing.

Specific Aim 1: Polypeptide Biodistribution and Pharmacokinetics

Specific Aim 1 of the proposal is to quantitate the *in vivo* distribution of CPP-ELP-H1 in normal and neoplastic tissue in an athymic tumor-bearing mouse model of breast cancer. After IV (intravenous) administration of radiolabeled polypeptides, we proposed to determine the plasma kinetics and the polypeptide concentration in normal and neoplastic tissue by quantitative autoradiography (QAR). We proposed to test three CPPs in order to optimize tumor targeting and minimize distribution in normal tissues.

Using the *in vitro* data described above and in Appendix 1, we decided to begin *in vivo* testing with Bac-ELP-H1 as the lead polypeptide. We first established MCF-7 tumors in nude mice by injection of tumor cells into the mammary fat pad, and tumor growth was enhanced by stimulation with 17-β-estradiol released at a controlled rate from a subcutaneously implanted pellet. Each animal was implanted with two tumors, one of which was heated, and one which served as an unheated control.

Tumor Heating Using Infrared Light. In the original proposal, we planned to heat tumor by immersing them in a warm water bath. Since that time, we have optimized a more efficient and specific heating method which employs infrared (IR) light to heat the tumors. The animal is anesthetized by isoflurane inhalation, and the tumor area is illuminated with IR light (805 nm) generated from the LED source of a Laser Systim 540 (Mettler Electronics). The tissue absorbs the light, which results in heating. In order to test the effectiveness of using IR light to heat the tumors and to determine the light intensity parameters required, three representative tumors (approximately 8 x 5 mm) were used in a heating trial. A needle thermocouple was placed in the tumor core, and the LED probe was positioned approximately 1 mm from the skin over the tumor site. The area surrounding the tumor was shielded from illumination using a non-transparent paper covering. The LED unit was activated at full pulse frequency of 10 kHz, and the tumor core temperature was monitored every minute. The tumor core reached the desired hyperthermia temperature within 20 minutes of the start of illumination, and the temperature remained stable at an average of 41 ºC for the remainder of an hour (Figure 1). The mouse body temperature rose slightly during the heating period as determined by a rectal temperature probe. However, this rise is likely an artifact of the placement of the LED probe. When positioned to heat the tumor, the LED probe is very close to the position of the rectal thermometer, likely leading to a falsely high temperature reading. Interestingly, the skin temperature at the illuminated site did not increase during the heating period. This likely reflects the fact that heating of the tissue results from absorbance of the IR light, so the heat generated by this device is centered deeper in the tissue with little temperature change at the skin surface. When the LED illumination was removed, the tumor tissue rapidly returned to baseline within 5 minutes.

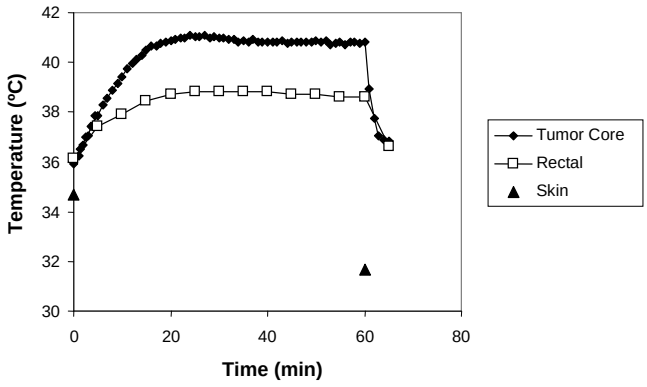

Figure 1. MCF-7 Tumor Heating by Infrared LED Light. MCF-7 tumors, an average of 8 mm x 5 mm, were heated for 1 h using LED generated IR light at 805 nm and 10 kHz pulse frequency. Tumor temperature was measured using a needle thermocouple inserted into the tumor core. Body temperature was monitored with a rectal probe, and the slight elevation seen was due to the proximity of the LED to the rectal probe. Skin temperature at the heated site was monitored using an IR beam temperature probe before and after the heating period. Data represent an average of 3 tumors; error bars were omitted for clarity.

Therefore, all future animal experiments will use IR light rather than a water bath for tumor hyperthermia. It is important to note that, while ideal for animal studies, this method is not practical in the clinical setting for heating tumors deep inside tissues. In the clinic, mild hyperthermia can be applied using high intensity focused ultrasound (HIFU) (reviewed in [10-12]), which can be focused to a very defined area and applied at deep depths. HIFU technology is currently available in the clinical setting.

Plasma Clearance of Bac-ELP-H1. The clearance rate of Bac-ELP-H1 was determined by injecting ^{125}I – labeled polypeptide into nude mice bearing MCF-7 tumors. Both the intravenous (i.v.) route and the intraperitoneal (i.p.) route were examined. The IP route was examined because of the ease of administering multiple injections if deemed necessary for tumor size reduction. When injected i.v., the plasma levels initially showed a rapid drop to about 50 % of the initial level within 60 min of the injection. However, after the first hour, the plasma level was quite stable (Figure 2). This long half life is typical of macromolecular carriers [13, 14], and may represent an advantage for delivery of therapeutic peptides, which are normally cleared or degraded quite quickly [15]. When injected i.p., the plasma levels were initially very low. However, with time the polypeptide did move from the lymphatic system into the blood circulation, and beginning at 2 h after injection, the i.p. route produced nearly the same plasma level as did the i.v. injection.

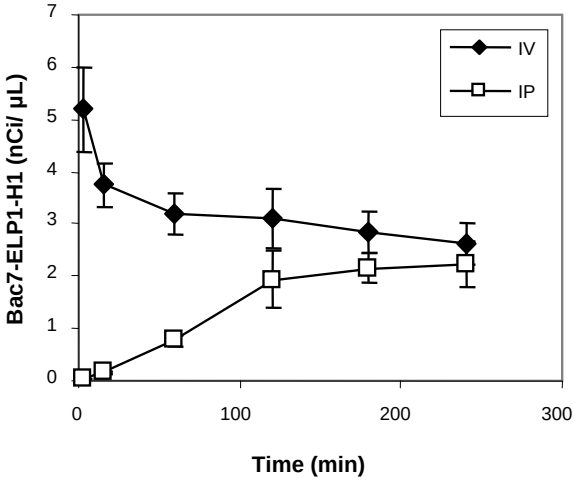

Figure 2. Plasma Clearance of Bac-ELP1-H1. 50 µCi of ^{125}I-labeled protein was injected IV via the jugular vein or IP in MCF-7 bearing mice. Blood was collected at the indicated times, centrifuged, and a sample of the plasma was counted using a γ counter. Data represent the average of 3 animals per injection route; error bars, SD.

Biodistribution of Bac-ELP-H1 in Nude Mice. The biodistribution of ^{125}I-Bac-ELP1-H1 was determined in the major organs of MCF-7 bearing mice 4 h after injection by γ counting (Figure 3). The polypeptide accumulated most highly in the liver. This is commonly seen with large molecular weight molecules [16], and is likely due to the high permeability of capillary beds in the liver to large molecular weight molecules [17]. High levels of Bac-ELP-H1 were also present in the spleen, kidneys, and lungs. A low level of Bac-ELP-H1 was found in the muscle beneath the tumor site, with a slight increase on the heated side. The polypeptide was found at low levels in the heart, and very little was found in the brain. The polypeptide distribution following the i.p. and i.v. injection routes did not differ widely. The only significant differences were a lower accumulation in the liver and lungs following the i.p. injection. This data suggests that the i.p. route may be a feasible mechanism for delivery of Bac-ELP-H1, but more data is needed to determine the stability of the polypeptide after each injection protocol.

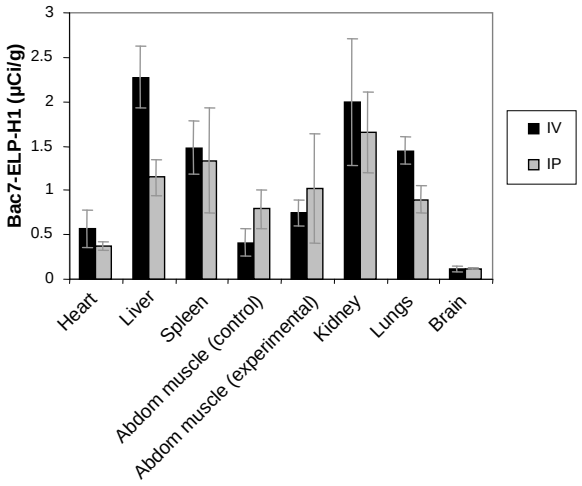

Figure 3. Biodistribution of Bac-ELP1-H1 in MCF-7 Tumor Bearing Mice. Mice were injected IV or IP with 50 µCi of ^{125}I-labeled protein. For IV injections, tumors were heated for 60 min after injection using an IR LED. For IP injections, tumors were heated from 60 min to 120 min after injection. 240 min after injection, the mice were sacrificed and samples of the major organs were weighed and counted using a γ counter. Data represent the average of 3 animals for each injection route; error bars, SD.

Accumulation of Bac7-ELP-H1 in MCF-7 Tumors. Tumor accumulation of ^{125}I-Bac-ELP-H1 was also determined. Mice bearing two MCF-7 tumors were injected i.v. with radiolabeled Bac-ELP1-H1. One tumor was heated for 1 h using the IR illumination technique described above. 4 h after injection, the animals were sacrificed, and the tumors were dissected and rapidly frozen. Tumors and radioactive standards were serially sectioned using a cryomicrotome, and tumor sections and standards were mounted onto slides and exposed to film to create autoradiograms. The films were digitized and analyzed using NIH Image. A standard curve of O.D. versus radioactivity was plotted for each film. The O.D. of 25 to 30 sections from each tumor were quantified, averaged, and fit to the standard curve to determine the amount of radioactivity in the tumor. This value was corrected for variations in injection amount for each animal, and an average of three animals was taken. 12 tumor slices from representative unheated (Figure 4A) and heated (Figure 4B) tumors are shown, and the average tumor uptake is shown in Figure 4C. The heated tumor accumulated 1.5 fold more Bac-ELP1-H1 than the nonheated tumor in the same animal. In addition, it is clear from the autoradiograms that the polypeptide is well distributed throughout the tumor. This is an important observation, as many large solid tumors are impermeable to high molecular weight macromolecules [18].

Protocol Improvements, Changes to the Originally Proposed Studies, and Future Work. Carrying out the biodistribution experiment revealed some problems with the approach and some areas where protocol

improvements could be made. The first problem that occurred concerned the method of labeling the protein. At the end of the 4 h plasma clearance / biodistribution experiment, 30 to 40 % of the radioactive label was present as free ^{125}I. This results from separation of the label from the protein, and results in inaccurate plasma clearance rates (since the free iodine clears much faster than the labeled protein) and inaccurate tumor and organ biodistribution. We identified this as a potential problem in the original proposal, and we have developed two alternative labeling methods that both result in a more stable attachment of label to protein. One option is to label the protein with ^{14}C, which is accomplished by expressing the protein in bacteria growing in minimal media with ^{14}C-glucose as the sole carbon source. We have optimized this labeling protocol for a related project, and, since the radioisotope is incorporated throughout the protein, there is no problem with separation of the label. Another alternative is to label the protein with fluorescent probes, and use fluorescence detection methods to monitor plasma clearance and tissue uptake. As shown below, we have used the fluorescence method in a recent experiment, and there is no problem with label separation and tissue detection and quantitation is possible.

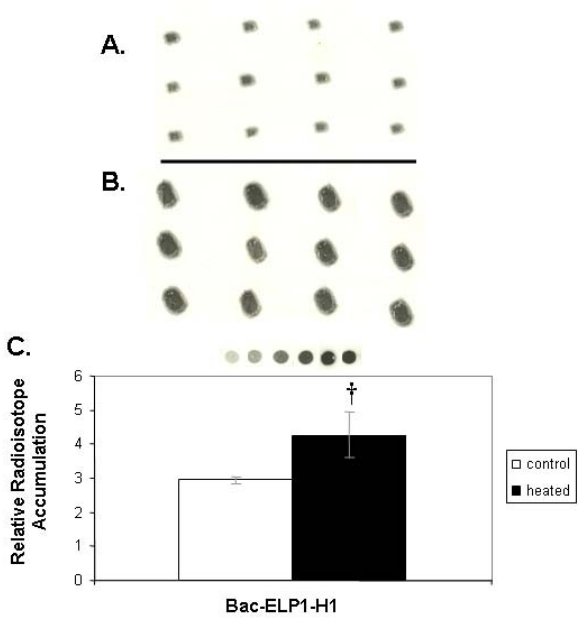

Figure 4. Accumulation of Bac-ELP1-H1 in MCF-7 Tumors. ^{125}I-Bac-ELP1-H1 was injected IV into Athymic nude mice bearing two MCF-7 tumors . One tumor was heated to a core temperature of 42 ºC for 1 h after injection, while the second was left at normal body temperature. The animal was sacrificed and the tumors frozen 4 h after injection. The tumors were serially sliced and exposed to film, and images were digitized and quantitated using NIH Image. Unheated (**A.**) and heated (**B.**) tumors from a representative animal are shown. 25 to 30 tumor slices from each animal were averaged, and data from 5 animals was pooled (**C.**), bars SEM. † Difference between heated and unheated tumors is statistically significant as assessed by a Student's t-test, $p < 0.05$.

A second area where we saw room for improvement was in the protocol used for tumor hyperthermia. Dreher et al. recently published the observation that cycling the tumor tissue between hyperthermia and normothermia resulted in enhanced accumulation of ELP as compared to one continuous hyperthermia session [19]. This is due to the fact that hyperthermia induces aggregation of ELP in the blood, and upon the removal of hyperthermia, the aggregated ELP re-dissolves, generating a concentration gradient from the blood to the tumor parenchyma. At this point, the soluble polypeptide diffuses down the concentration gradient into the tumor's extravascular space. In a related study, we have also shown that the hyperthermia cycling protocol is more effective than continuous heating for tumor targeting, resulting in a higher and more sustained accumulation of ELP in the heated tumor. Therefore, a heat cycling protocol in which the tumor is raised to >40 ºC for 20 min, then cooled to body temperature for 10 min, and repeated for four cycles, will be used for all future hyperthermia applications in this project.

The third area of improvement involves the animal model used. While the MCF-7 human xenograft model was proposed and used in the original experiment, we have recently realized that it may not be the best model for initial screening for therapeutic efficacy. The problems with the MCF-7 xenograft model include the need to use immunocompromised animals and the unpredictable and often poor tumor growth achieved. When discussing these issues with several senior investigators at the 2009 LINKS meeting, I was encouraged to use a syngeneic mouse model of breast cancer for initial screening. This model involves the use of E0771 cells, which are an estrogen receptor positive mouse breast tumor cell line that are syngeneic with C57BL/6 mice. There are several advantages to this model over the MCF-7 xenograft model. First, the tumors grow very predictably and very aggressively, elimination the reproducibility problems encountered with the poorly growing MCF-7 tumors and requiring fewer animals to obtain significant data. Second and most important, the tumors grow in mice with fully functioning immune systems. This eliminates the need to do toxicity and immunogenicity studies in a separate experiment (as proposed in the original application) since the data can be collected simultaneously with the tumor size reduction data. Also, evaluation of therapeutic efficacy in an immunocompetent animal is important since induction of an immune response by the therapeutic agent, especially for peptide therapies, can be a major hurdle to effective and safe therapy. Use of the E0771 syngeneic model will allow us not only to collect biodistribution and tumor reduction data, but it will elucidate any issues of side effects or loss of therapeutic efficacy resulting from immunogenicity of the therapeutic peptides. Therefore, both Specific Aims 1 and 2 of the original proposal will be carried out using the E0771 model as the main cancer model. Once a therapeutic polypeptide is validated in this model, MCF-7 xenografts can be used for final confirmation of tumor reduction efficacy against a human cell line.

In summary, the originally proposed approach has been modified to include fluorescent labeling of the polypeptide, tumor thermal cycling, and the E0771 syngeneic breast tumor model. Using these changes, we recently carried out another biodistribution experiment.

Plasma Clearance, Tumor Uptake, and Biodistribution of Bac-ELP-H1 in E0771 Breast Tumors. C57BL/6 were implanted with E0771 tumors in the mammary fat pad. An arterial line was placed in the femoral artery for blood sampling, and Rhodamine-labeled Bac-ELP1-H1 was injected IV via the femoral vein. In the heated group, the tumor was heated to >40 ºC for 20 min, allowed to cool to body temperature for 10 min, and this cycling protocol was repeated for 4 cycles. In the control group, the tumor was left at body temperature. Blood was sampled at the indicated time points, and, 3 h after injection, the animal was euthanized, and the tumor and all major organs were rapidly frozen. Plasma samples were analyzed for fluorescence intensity using an Aminco Bowman Series 2 luminescence spectrometer, and separation of label from the polypeptide was assessed by TCA precipitation. Tumors and organs were cut into 20 µm sections using a cryomicrotome, and sections were mounted onto slides. Slides were scanned using a Packard Bioscience ScanArray Express microarray scanner. A standard curve was made by mixing known amounts of the same batch of protein used for animal injections with liver homogenate, drawing the mixture into a 1 cc syringe, rapidly freezing, and cutting into 20 µm sections. All tissue slides and standards were imaged using the same laser power and PMT voltage to allow equal comparison of all samples.

As shown in Figure 5A, approximately 33% of the Bac-ELP1-H1 cleared from the plasma during the first hour, and the remainder of the protein was very stable, clearing at a slow rate. Over 40% of the injected protein was still present in circulation 3 h after injection. Heating the tumor did not have a significant effect on plasma levels. At the end of the experiment, only an average of 4.5 % of the label was present as free label, indicating that the rhodamine - labeled protein is more resistant to loss of label than the ^{125}I labeled – protein used above. Figure 5B shows a sample of a slide containing ten sections of an unheated E0771 tumor after scanning using the ScanArray Express. This data is currently being analyzed. The fluorescence intensity will be determined for 100 sections of each tumor. The fluorescence data will be averaged for each tumor and fit to a standard curve to determine the mg of protein / mg of tissue. These values will be averaged across all tumors in each group to determine whether hyperthermia effectively increased tumor uptake of the polypeptide.

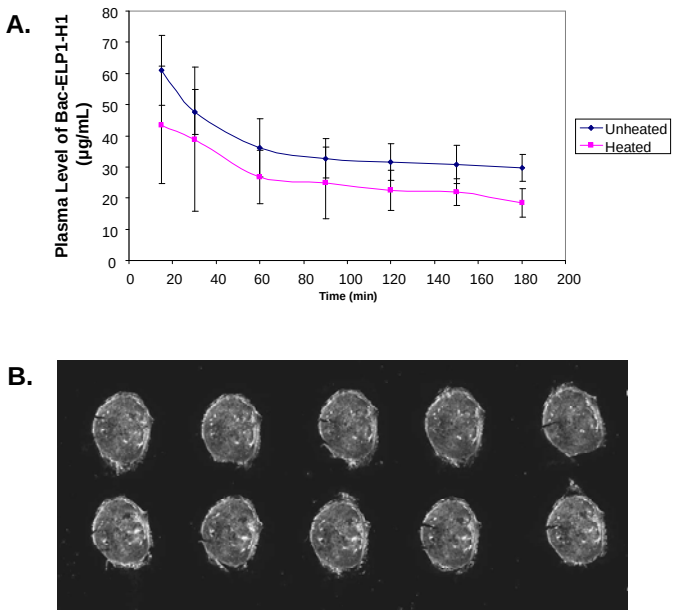

Figure 5. Plasma Clearance and Tumor Distribution of Bac-ELP1-H1 in E0771 Bearing Mice. Mice were injected IV with 100 mg/kg of rhodamine-labeled protein. Heated tumors were heated by increasing the temperature to >40 ºC for 20 min, followed by 10 min of cooling to body temperature, and this heat cycling was repeated for 4 cycles. Blood was sampled at the indicated times. 180 min after injection, the mice were sacrificed and the tumor and major organs were frozen and sectioned. **A.** Plasma data was fit to standard curves. Data represent the average of 3 animals for each injection route; error bars, SD. **B.** A sample of the fluorescence scan of a slide of unheated tumor sections. The fluorescence intensity of each tumor will be fit to a standard curve and expressed relative to the plasma levels for each animal.

As stated in the original proposal, this analysis will be carried out for three CPP-ELP-H1 constructs. We began with the Bac CPP because it was most potent *in vitro*, but all will be evaluated *in vivo* because each CPP may behave differently and have different tumor distributions. We will also perform this experiment using the non-thermally responsive ELP2 – containing polypeptides as controls to parse the effects of ELP aggregation from nonspecific effects of hyperthermia. The IP route will also be examined. In addition to analysis of total tumor fluorescence, animals will be injected with high molecular weight FITC – dextran as described in the original proposal to mark the perfused vasculature, and the distribution of the protein will be examined within the tumor relative to the dextran signal. This data will allow comparison of each CPP and comparison of hyperthermia versus control for amount of extravasation of the polypeptide within the tumor.

Specific Aim 2: Tumor Size Reduction

As a preliminary test for efficacy of the Bac-ELP-H1 polypeptide, E0771 tumors were grown in the mammary fat pad, and polypeptide was injected daily. The IP route was used to accommodate multiple treatments. 200 mg/kg was chosen as a starting dose, and animals were treated daily for seven days. No heat was used in the initial experiment. As shown in Figure 6A, daily injection of Bac-ELP1-H1 caused significant reduction in the tumor proliferation rate. The control polypeptide Bac-ELP1, which lacks the c-Myc inhibitory peptide, had no effect on tumor size or growth rate, indicating that the c-Myc inhibitory peptide was the active moiety in the construct. The animals' body weight was monitored as an indicator of overall treatment toxicity. As shown in Figure 6B, the animals' body weight dropped during the first two days of injections, but began to rapidly increase following the initial drop. By the end of the experiment, the treated animals had nearly recovered to the average body weight of the control group. No other signs of acute toxicity were observed. Interestingly, the animals treated with the Bac-ELP1 control showed a similar initial decrease in body weight to the Bac-ELP1-H1 animals, indicating that the weight loss may be induced by the Bac peptide or the ELP polypeptide, not the c-Myc inhibitory portion.

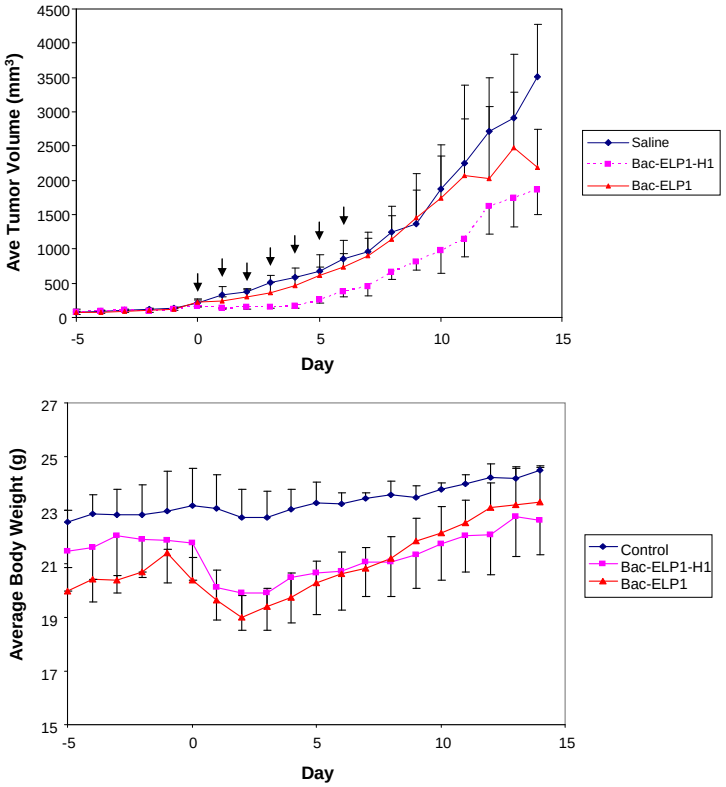

Figure 6. A. Tumor Reduction with Bac-ELP1-H1. C57BL/6 mice bearing E0771 mammary fat pad tumors were injected on the indicated days (↓) with 200 mg/kg Bac-ELP1-H1 by IP injection. Tumor size was monitored daily by caliper measurement. **B.** Body weight of animals was monitored daily during and after the treatment period. n = 5 animals per group; bars, SD.

With the encouraging result of slowed tumor progression observed, we next tested whether combination of Bac-ELP1-H1 treatment with hyperthermia could further reduce tumor size. Bac-ELP1-H1 (200 mg/kg) was injected as above, except treatment was administered every other day. In the heated group, hyperthermia was administered to the tumor beginning 1 h after the injection (to allow time for the polypeptide to reach a high plasma concentration) using the heat cycling protocol described above. As shown in Figure 7, tumors treated with Bac-ELP1-H1 and hyperthermia actually decreased in size the day after the first and second treatments. However, the tumors were able to recover and continue to grow after treatment. In the unheated group, little effect was seen on tumor size using this schedule of treatment. Based on these data, we believe that, with alterations in treatment dose and schedule, significant tumor reduction, and possibly even complete tumor regression can be achieved with a combination of Bac-ELP1-H1 and hyperthermia. Future experiments will employ daily treatments in an attempt to maximize the tumor reduction effect, and control experiments will be carried out with the Bac-ELP1 and Bac-ELP2-H1 polypeptides. Also, other CPP-ELP-H1 constructs may be tested if they prove advantageous in Specific Aim 1 testing. Finally, detailed toxicity and immunogenicity studies will be performed as outlined in the original proposal.

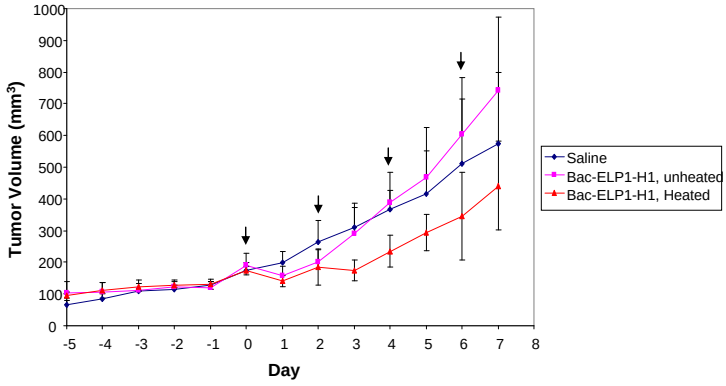

Figure 7. Tumor Reduction with Bac-ELP1-H1 and Hyperthermia. C57BL/6 mice bearing E0771 mammary fat pad tumors were injected on the indicated days (↓) with 200 mg/kg Bac-ELP1-H1 by IP injection. Heated tumors were heated by increasing the temperature to >40 ºC for 20 min, followed by 10 min of cooling to body temperature, and this heat cycling was repeated for 4 cycles. Tumor size was monitored daily by caliper measurement. n = 5 animals per group; bars, SD.

Key Research Accomplishments

- Specific Aim 1: Plasma Clearance and Biodistribution of ELP – fused c-Myc Inhibitory Polypeptides.
    - Performed plasma clearance and tumor and organ distribution of polypeptide labeled with ^{125}I in the MCF-7 xenograft model.
        - Found that the polypeptide exhibited a long plasma half – life, and that the IP route was a viable option for polypeptide delivery.
        - Demonstrated a 1.5 fold enhancement of tumor uptake of the polypeptide with a 1 h hyperthermia treatment.
        - Identified problems with the labeling method used, and made changes to the protocol to address these problems and to incorporate improvements in the approach. These changes include using thermal cycling for tumor hyperthermia and using a syngeneic breast cancer model for initial polypeptide screening.
    - Performed plasma clearance and tumor and organ distribution of the polypeptide labeled with rhodamine in the E0771 syngeneic model.
        - Found that the polypeptide exhibited a long plasma half – life, and confirmed that fluorescent labeling was more stable than ^{125}I labeling.
        - Analysis of tumor and organ distribution is currently ongoing.
    - Future experiments will examine three CPPs fused to the ELP-H1 polypeptide, and will test the ELP2 – containing control polypeptide. Intratumoral distribution will also be carefully examined.
- Specific Aim 2: Breast Tumor Reduction using CPP-ELP-H1 polypeptides.
    - Performed a pilot tumor reduction study using Bac-ELP1-H1, injected daily, without hyperthermia.
        - Demonstrated a slowed progression of tumor volume with the active Bac-ELP1-H1 polypeptide, and no effect from the Bac-ELP1 control polypeptide.
    - Performed a pilot tumor reduction study using the Bac-ELP1-H1 polypeptide in combination with hyperthermia.
        - Demonstrated tumor regression in the days following the first two treatments with Bac-ELP1-H1 and hyperthermia.
    - Future experiments will optimize the dose and treatment schedule to maximize the tumor reduction effect of Bac-ELP-H1. Other CPPs will also be tested if they show promising biodistribution. The ELP2 – containing control polypeptides will be tested. Detailed toxicity and immunogenicity studies will be carried out.

Reportable Outcomes

An *in vitro* comparison of the Bac, Tat, and Pen CPPs was completed prior to beginning the proposed research. These data have been published recently [20], and a copy of the manuscript is enclosed in Appendix 1. These results were used to guide the studies described above.

The *in vivo* results shown in this report will be published as an abstract and presented in poster form at the 2010 annual meeting of the American Association for Cancer Research (AACR) in April in Washington, DC.

Conclusions

During the first year of funding, we have made significant progress toward accomplishing the goals outlined in this award proposal. The biodistribution study has begun with promising results, and refinements to the protocol have greatly improved the approach and the chance for generation of meaningful data. Also, pilot studies for the tumor reduction experiments have demonstrated that the polypeptides under development do have antitumor activity. Future experiments will refine the dose and treatment schedule necessary to achieve sustained tumor reductions or even complete regression. Once the proposed studies are completed, we will have the efficacy and toxicity data necessary for advancing this technology toward the next phase of clinical development.

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Appendix 1

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Targeting a c-Myc inhibitory polypeptide to specific intracellular compartments using cell penetrating peptides

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ABSTRACT

The therapeutic index of current anti-cancer chemotherapeutics can be improved by two major mechanisms: 1) developing drugs which are specifically toxic to the cancer cells and 2) developing methods to deliver drugs to the tumor site. In an attempt to combine these approaches, we developed a thermally responsive polypeptide inhibitor of c-Myc. This polypeptide is based on the thermally responsive Elastin-like polypeptide (ELP). When injected systemically, ELP-fused drugs will aggregate and accumulate at the tumor site where local hyperthermia is applied. ELP was fused to a peptide which blocks c-Myc/Max dimerization (H1), thereby inhibiting transcription activation by c-Myc (ELP-H1). In this study, the cellular uptake, intracellular distribution, and potency of the Pen, Tat and Bac cell penetrating peptides fused to ELP-H1 were evaluated. While Pen-ELP-H1 and Tat-ELP-H1 were localized in the cytoplasm, Bac-ELP-H1 localized to the nucleus in a subset of the cells and was the most potent inhibitor of MCF-7 cell proliferation. This data demonstrates that ELP can be targeted to the desired cellular compartment simply by choice of the CPP used, resulting in a more potent nuclear targeted c-Myc inhibitory polypeptide which may be beneficial in cancer therapy.

1. Introduction

Classical approaches for cancer chemotherapy utilize systemic delivery of highly toxic compounds and are often limited by poor efficacy and/or severe side-effects. One approach to combat this problem is the development of drug carriers capable of being targeted to the tumor site. This may be achieved by using macromolecular drug carriers, which have the advantages of extending drug half lives [1] and accumulating in tumor tissue [2–5]. This approach leads to specific drug delivery to the tumor, resulting in improved efficacy and reduced systemic toxicity. A second approach is the development of drugs that are only toxic to the tumor cells, while sparing the non-cancerous tissue. Of many developmental classes of this type of drug, one emerging approach is the use of bioactive peptides. Several classes of peptides have been discovered that bind and inhibit oncogenes [6], cause cell cycle arrest [7], or induce apoptosis [8]. These peptides are attractive from a drug development standpoint because they are specific for their intracellular target, and can therefore be designed to specifically inhibit the proliferation of cancer cells. However, their utility is limited by their poor pharmacokinetic parameters. Peptides are easily degraded in circulation, poorly deposited in tumor tissue, and inefficiently internalized by tumor cells [9,10]. In an attempt to overcome these limitations and combine the advantages of tumor-specific inhibitory peptides and macromolecular carriers, we have developed a thermally responsive polypeptide vector for bioactive peptide delivery.

The thermally responsive peptide delivery vector is based on Elastin-like polypeptide (ELP). ELP is an approximately 60 kDa polypeptide made of repeating units of the pentapeptide VPGXG, where X can be any amino acid except proline [11]. ELP is soluble below a characteristic transition temperature (T_t), but undergoes an inverse phase transition when the solution temperature is raised above the T_t [11,12]. This phase transition leads to aggregation of the polypeptide into first 100 nm sized particles, then, with further heat, into micron sized aggregates [13]. This property can be exploited for drug delivery by attaching a therapeutic molecule to ELP [14–17]. When injected systemically, ELP will remain soluble and eventually be cleared from circulation at normal body temperature, but it will aggregate and accumulate at a tumor site where local mild hyperthermia is applied [18–20]. ELP is advantageous as a drug carrier because it is a macromolecule, which confers the advantages of increased drug solubility [21], extended plasma half life [1], passive tumor accumulation [4], and reduced drug toxicity [22,23], and because it is thermally responsive, which adds the additional advantage that it may be actively targeted to the tumor tissue.

In order to enhance uptake of ELP-based polypeptides, we fused them with cell penetrating peptides (CPPs). CPPs are short peptides known to enhance the cellular uptake of large cargo. The three CPPs used in this study are the penetratin (Pen) peptide from Antennapedia [24], the Tat peptide from the HIV-1 Tat protein [25], and the Bac peptide from the bovine antimicrobial bactenecin peptide Bac 7

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[26]. Pen and Tat are widely used for membrane translocation and have been fused to various types and sizes of cargo ranging from small molecule fluorochromes to proteins, synthetic macromolecules, antisense oligonucleotides, and liposomes [27–32]. The cell penetrating properties of the Bac peptide were discovered more recently [26,33]. The Bac 7 peptide was first purified from bovine neutrophils due to interest in its use as an antimicrobial agent [34]. More recently, its was published that amino acids 1–17 and 46–59 bind acidic phospholipids [33], and amino acids 1–24 were shown to be cell permeable [26]. Sadler et al. [26] further concluded that the Bac 7 1–24 peptide traversed the cell membrane and resided inside the nucleus of murine monocytes, and they first demonstrated the peptide's use as a cell penetrating peptide. A biotinylated version of the Bac 7 1–24 peptide was capable of enhancing the uptake of avidin over 10 fold in murine monocytes as compared to avidin alone. This work was extended recently by Tomasinsig et al. [35], who found that a peptide composed of amino acids 1–35 of Bac 7 was taken up by 3T3 and U937 cells in a non-toxic, energy and temperature dependent manner.

In a previous study, we made fusion polypeptides of ELP with the CPPs Pen, Tat, and the hydrophobic membrane translocating sequence (MTS) from Kaposi fibroblast growth factor [17]. All three CPPs enhanced cellular uptake relative to ELP alone, with the Pen peptide causing the most enhancement. All CPP-ELPs tested entered the cells in an energy dependent manner and localized to the cell cytoplasm.

In a separate previous study, ELP was used for delivery of a peptide inhibitor of c-Myc transcriptional activation (H1-S6A, F8A) [14]. This peptide, first described by Draeger and Mullen [36], was designed by modification of helix 1 (H1) of the basic-helix-loop-helix-zipper domain (bHLH-zip) of c-Myc. The H1-S6A, F8A peptide was shown to block DNA binding by the C-terminal 92 amino acids of c-Myc, presumably by binding the bHLH-zip domain of c-Myc [36]. Giorello et al. extended this work by fusing the H1-S6A, F8A peptide with Pen [6]. This fusion peptide was capable of inhibiting co-immunoprecipitation of c-Myc and its dimerization partner Max, blocking transcription of c-Myc responsive genes and inhibiting proliferation in MCF-7 breast cancer cells. A retro-inverso (RI) form of the Pen-H1-S6A, F8A peptide was shown to be 5 to 10 times more potent for inhibition of cell proliferation [37]. Additionally, this RI-Pen-H1-S6A, F8A peptide was found to disrupt the interaction between c-Myc and INI1 [38,39], a subunit of the SWI/SNF complex and component of the enhanceosome [40], and the authors propose this interaction as an alternative mechanism of proliferation inhibition by the RI-Pen-H1-S6A, F8A peptide [39].

Previously, we conjugated the H1-S6A, F8A peptide and the Pen peptide to ELP for thermally targeted delivery (Pen-ELP-H1) [14]. Uptake of Pen-ELP-H1 in MCF-7 cells was increased by both the Pen peptide and by the hyperthermia-induced aggregation of ELP. It was demonstrated that Pen-ELP-H1 could sequester endogenous c-Myc to the cytoplasm, thus preventing its interaction with Max or other nuclear partners and its activation of transcription. The result of this inhibition was reduction in the proliferation rate of the MCF-7 cells. In addition, Pen-ELP-H1 increased the potency of topoisomerase II inhibitors, demonstrating potential utility for combination therapy [41]. However, after a short exposure to Pen-ELP-H1, inhibition of cell proliferation was observed 11 days later. In an attempt to increase the potency of the ELP-H1 polypeptide, it was fused to the Tat or Bac CPPs. Here we report cellular uptake, subcellular distribution, and proliferation inhibition for each construct.

2. Materials and methods

2.1. Cell culture

MCF-7 human breast carcinoma cells were cultured in Minimal Essential Medium supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, Sacramento, CA), 1 mM sodium pyruvate, BME amino acids, 5 μg/ml insulin (Sigma, St. Louis, MO), 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml amphotericin B (Invitrogen, Carlsbad, CA). Cultures were maintained at 37 °C in a humidified atmosphere+5% CO_2.

2.2. ELP synthesis

pUC19-ELP1 and pUC19-ELP2 were synthesized by recursive directional ligation as described previously [42]. The ELP coding sequence was modified by the addition of the penetratin peptide (RQIKIWFQNRRMKWKK), the Tat peptide (YGRKKRRQRRR), or the Bac peptide (RRIRPRPPRLPRPRPRPLPFPRPG) to the N-terminus of ELP and the c-Myc inhibitory peptide H1-S6A, F8A (NELKRAFAALRDQI) to the C-terminus using the method described by Meyer and Chilkoti [42]. The resulting sequence was then confirmed by DNA sequencing. Modification of ELP with drugs or peptides can change the T_t [15,43]. The T_t of ELP is dependent on the amino acid composition at the X position and on the polypeptide's molecular weight. Therefore, a library of ELP molecules of various molecular weights was synthesized using the recursive directional ligation method [42]. The new ELP library contained the VPGXG sequence with the amino acids V, G, and A in a 3:1:1 ratio at the X position, respectively. This ELP was named ELPa. Various molecular weights of ELPs were generated by utilizing 5 to 80 VPGXG repeats, and the ELP was named descriptively (e.g. ELPa5, ELPa10, … ELPa80). In order to generate an ELP carrier with a suitable T_t for thermal targeting, the appropriate ELP moiety from this new library was used to adjust the T_t. In addition, in the case of Bac-ELP1-H1, the ELP1 moiety was shortened from 150 repeats to approximately 130 repeats in order to slightly increase the T_t (Bac-ELP1*-H1).

2.3. ELP purification

Pen-ELP1-H1, Tat-ELPa80-H1, and Bac-ELP1*-H1 were expressed using the pET expression system [44] and purified by inverse transition cycling as previously described [14]. Polypeptides were labeled with fluorescent dyes (5-iodoacetomidofluorescein or tetramethylrhodamine-5-iodoacetamide dihydroiodide; Molecular Probes, Eugene, OR) on a unique cysteine residue as described in [14].

2.4. Characterization of the transition temperature

Each polypeptide was heated at 1 °C/min in a multicell holder in a UV–visible spectrophotometer (Cary 100, Varian instruments). For comparison of all constructs, the T_t was determined at 10 μM polypeptide concentration in PBS. For determination of the useful concentration range of Pen-ELP1-H1, Tat-ELPa80-H1, and Bac-ELP1*-H1, the T_t was determined at concentrations from 1 to 30 μM in MCF-7 media containing 10% fetal bovine serum (FBS). The turbidity was monitored by measuring the absorbance at 350 nm every minute. Absorbance data was converted to a percentage of the maximum absorbance in order to display all concentrations on the same plot. The T_t was defined as the temperature at which polypeptide aggregation is 50% of the maximum. In order to determine the polypeptide concentration range in which the T_t lies between 37 and 42 °C, the T_t was plotted versus polypeptide concentration, and the data was fitted with a logarithmic equation.

2.5. Flow cytometry analysis of cellular uptake

MCF-7 cells were incubated with fluorescein-labeled polypeptides (10 μM) for 1 h at 37 or 42 °C. Cells were rinsed with PBS and removed from the plates using a non-enzymatic cell dissociation buffer (Invitrogen, Carlsbad, CA) to avoid degradation of polypeptide bound to the cell surface. Fluorescein fluorescence was measured using a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA). Forward versus

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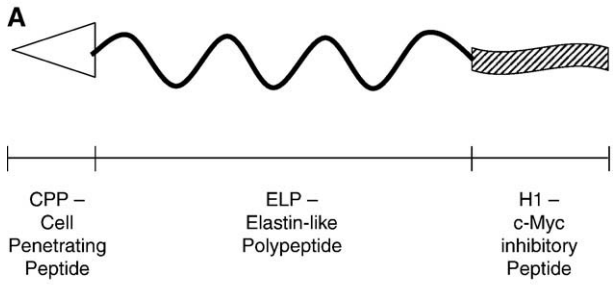

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CPP	Amino Acid Sequence
Pen	RQIKIWFQNRRMKWKK
Tat	YGRKKRRQRRR
Bac	RRIRPRPPRLPRPRPRPLPFPRPG

Fig. 1. Schematic representation of the ELP-based peptide delivery vector. A. The thermally responsive ELP polypeptide is fused at its N-terminus to a cell penetrating peptide (CPP) to mediate uptake of the macromolecule across the plasma membrane and dictate intracellular localization. At the C-terminus, a peptide which inhibits transcriptional activation by c-Myc and subsequently inhibits cell proliferation is added. B. Table of CPPs used in this study.

side scatter gating was used to remove cell debris from the analysis, and the mean cellular fluorescence intensity was recorded. The mean cellular fluorescence was corrected for differences in labeling efficiencies among polypeptides, and the results shown are an average of at least 3 experiments, bars, SEM. Results were analyzed using an ANOVA with a post-hoc Bonferroni multiple comparison, and significant differences ($p<0.01$, 95% CI) between each polypeptide above and below the T_t and between each CPP-ELP and ELP control are indicated.

2.6. Cell proliferation

For comparison of Pen-ELP1-H1, Tat-ELPa80-H1, and Bac-ELP1*-H1 potency, MCF-7 cells were plated in 96 well plates (2000 cells/well) and treated 24 h later (day 1) with each polypeptide for 1 h at 37 or 42 °C at a range of concentrations from 1 to 40 μM. After the 1 h treatment, cells were rinsed and returned to the cell culture incubator. Media was changed on day 3, and cell viability was determined on day 7 using the MTS cell viability assay (Promega, Madison, WI). Results represent the mean of at least 3 experiments, bars, SEM.

2.7. Fluorescence microscopy

In order to compare the intracellular localization of the polypeptides, MCF-7 cells were treated with rhodamine-labeled Pen-ELP1-H1, Tat-ELPa80-H1, or Bac-ELP1*-H1. Cells were plated on coverslips and exposed each polypeptide (20 μM) for 1 h at 37 or 42 °C. The cells were rinsed and returned to the incubator. Twenty-four hours after treatment, the cells were fixed with 4% paraformaldehyde (PFA), and the coverslips were mounted onto slides and imaged using a TCS SP2 laser scanning confocal microscope with a 100× oil immersion objective (Leica, Wetzlar, Germany). PMT voltages were adjusted to maximize image resolution and intensity. Therefore, the image intensity does not represent the amount of polypeptide in the cells.

For determination of the concentration dependence of Bac-ELP1*-H1 nuclear localization, MCF-7 cells were treated with rhodamine-labeled Bac-ELP1*-H1 for 1 h at 37 or 42 °C at concentrations ranging from 5–40 μM, then rinsed and returned to the incubator. Twenty-four hours after treatment, the cells were fixed with 4% PFA, costained with Hoescht 33342 (1 μg/ml) to elucidate the nuclei, and random fields were imaged using a Zeiss Axiovert 200 epifluorescence microscope. The Hoescht channel was used to scan for cells in order to avoid bias in the data. Approximately 100 cells were counted per sample, and the Bac-ELP1*-H1 localization was scored as nuclear or cytoplasmic. The percentage of cells with Bac-ELP1*-H1 in the nucleus was recorded for each sample, and the experiment was repeated 3 times. The data shown is the average of the 3 experiments, bars, SE. Results were analyzed using an ANOVA with a post-hoc Bonferroni multiple comparison, and significant differences ($p<0.01$, 95% CI) among all concentrations tested and between each concentration above and below the T_t are indicated.

For determination of the time dependence of Bac-ELP1*-H1 nuclear localization, MCF-7 cells were treated with 30 μM rhodamine-labeled Bac-ELP1*-H1 for 1 h at 42 °C. Cells were fixed 4 h, 24 h, 48 h, and 72 h after treatment, co-stained with Hoescht, and assayed for nuclear localization as described above.

In order to obtain high quality confocal images, the concentration and time-dependent nuclear localization experiments were repeated exactly as described above, but the cells were co-stained with Sytox green nucleic acid stain (25 nM for 10 min) and imaged using a Leica TCS SP2 laser scanning confocal microscope.

3. Results

3.1. Design of CPP-ELP-H1 polypeptides

Previous studies utilized Pen-ELP for thermally targeted delivery of a c-Myc inhibitory peptide [14,41]. In this study, two different CPPs were attached to the ELP-H1 polypeptide in an attempt to improve its potency by either enhancing the cellular uptake or by altering the intracellular localization. The design of the CPP-ELP-H1 constructs used in this study is shown in Fig. 1A. In addition to penetratin, the protein transduction domains from the HIV-1 Tat protein and the Bac CPP from the bovine antimicrobial Bac 7 peptide were attached at the N-terminus of ELP (Fig. 1B).

3.2. Thermal aggregation of CPP-ELP-H1 polypeptides

For drug delivery applications, the T_t of ELP should be above body temperature (37 °C) to avoid systemic aggregation but below 42 °C to avoid significant tissue damage by the hyperthermia. The T_t of ELP is dependent on its intrinsic properties, including the mole fraction and polarity of the amino acid in the guest "X" position in the VPGXG repeat and the ELP molecular weight; and on external factors, including the ELP concentration and the concentration of salts and other co-solutes [11,45,46]. The "X" amino acid and the ELP molecular weight can be easily controlled by manipulation of the ELP sequence [42], making the T_t "tunable" to any desired temperature. This fact is crucial in the use of ELP for delivery of peptides, because N- and C-terminal modifications of ELP with these peptides will alter the T_t [14,15,43]. Previous studies have used ELP1, which contains 150 VPGXG pentapeptides, where X is Val, Gly, and Ala in a 5:3:2 ratio, respectively [18]. Unmodified ELP1 has a T_t of 41 °C at 25 μM in PBS [18].

In order to determine the effect of the CPP and H1 peptide additions on the T_t, a turbidity profile was obtained by monitoring the

Table 1
Physical parameters of ELP-based polypeptides used in the study

Polypeptide	Number of VPGXG repeats	X amino acid ratio	Molecular weight (kDa)	T_t[a]
Pen-ELP1	150	V:G:A — 5:3:2	62.1	34.5
Pen-ELP1-H1	150	V:G:A — 5:3:2	63.4	40.0
Tat-ELP1	150	V:G:A — 5:3:2	61.3	46.7
Tat-ELP1-H1	150	V:G:A — 5:3:2	62.9	51.5
Tat-ELPa80	80	V:G:A — 3:1:1	40.8	39.7
Tat-ELPa80-H1	80	V:G:A — 3:1:1	42.4	39.3
Bac-ELP1	150	V:G:A — 5:3:2	62.9	44.0
Bac-ELP1*-H1	~130	V:G:A — 5:3:2	~57	42.9

[a] Transition temperature as determined at 10 μM protein concentration in PBS.

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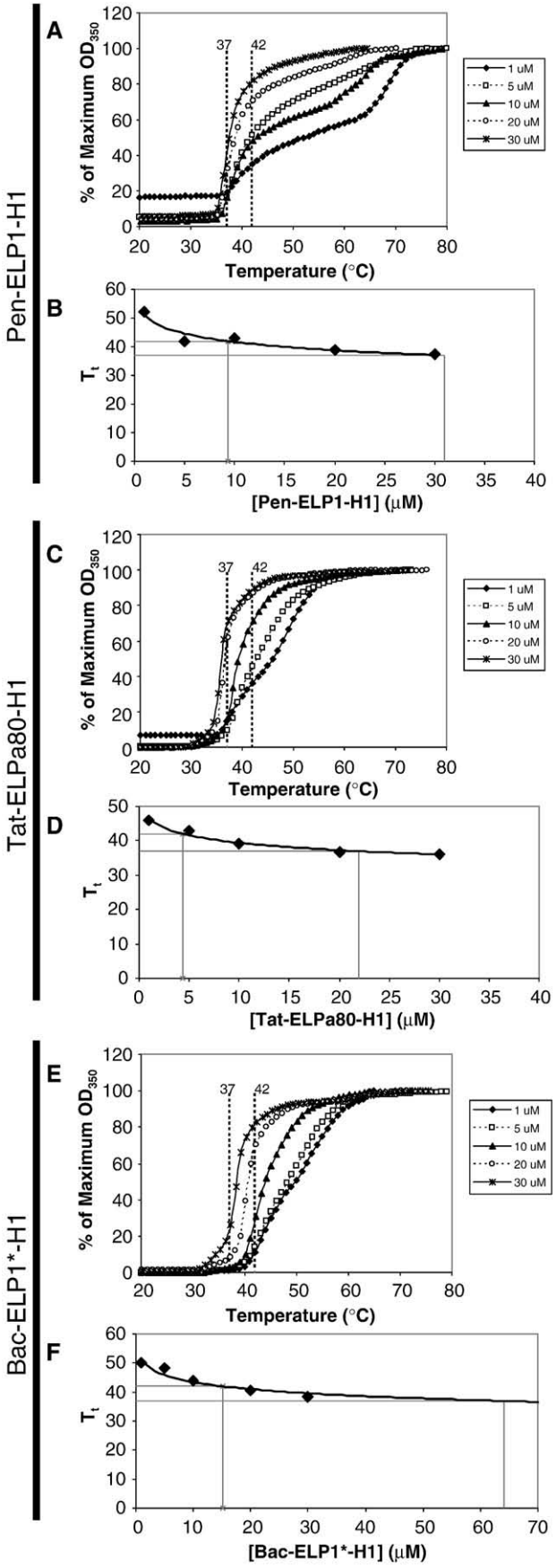

OD_{350} while heating solutions of each CPP-ELP-H1 polypeptide or control polypeptides lacking the H1 sequences. In order to generate standardized conditions under which all constructs could be compared, the T_t, or the point at which the aggregation was 50% of the maximum, was determined with each construct dissolved at 10 μM in PBS. Table 1 shows the makeup and number of VPGXG repeats, molecular weight, and T_t of each ELP-based polypeptide used in this study. Of the CPP-ELP-H1 polypeptides, Pen-ELP1-H1 and Bac-ELP1*-H1 (containing an ELP1 moiety shortened by approximately 20 VPGXG repeats) had transition temperatures in a suitable range for thermal targeting. Tat-ELP1-H1, however, had a T_t of 51.5 °C, far too high for thermal targeting. Therefore, for the Tat-containing polypeptide, the ELP1 moiety was replaced with ELPa80. This change caused a shift in the T_t to 39.3 °C, a suitable T_t for thermal targeting. Therefore, Tat-ELPa80-H1 was used for the remainder of experiments in this study.

As stated above, the T_t is affected by the ELP concentration and the concentration of other co-solutes. In order to accurately determine the T_t of the constructs under experimental conditions, aggregation curves were collected for various concentrations of each polypeptide dissolved in MCF-7 cell culture media containing 10% FBS. The absorbance data was converted to percentage of maximum aggregation in order to easily view all concentrations on the same plot. Pen-ELP1-H1 had a T_t suitable for thermal targeting in the concentration range tested. Fig. 2A shows that Pen-ELP1-H1 began to aggregate near 37 °C, and significant aggregation was observed at 42 °C at all concentrations. The T_t was inversely dependent on the polypeptide concentration and was well described by a logarithmic relationship (Fig. 2B.) Fitting the concentration versus T_t data showed that, for concentrations between 9.3 and 31 μM Pen-ELP1-H1, the T_t lies between 37 and 42 °C. This is the concentration range useful for treatment of cells with Pen-ELP1-H1. Some thermal effects may also be observed at concentrations lower than 9.3 μM because some aggregation will occur at the hyperthermia temperature, and experiments conducted above 31 μM must be interpreted with the consideration that aggregation of the polypeptides has begun at the normothermia temperature.

This experiment was repeated with Tat-ELP1-H1, and it was found that the T_t was higher and above the desired hyperthermia temperature. At 10 μM in PBS, the T_t of Tat-ELP1-H1 was 51.5 °C (Table 1). Therefore, the ELP1 moiety was replaced with ELPa80, which contains 80 VPGXG repeats with the "X" position occupied by Val, Gly, and Ala in a 3:1:1 ratio, respectively. Tat-ELPa80-H1 did have a suitable aggregation profile for hyperthermia treatments (Fig. 2C), and its T_t was between 37 and 42 °C between 4.4 and 22 μM, respectively (Fig. 2D). Finally, Bac-ELP1*-H1 was tested using the same assay. In order to increase the T_t into the therapeutic range, the ELP1 moiety was shortened from 150 VPGXG repeats to approximately 130 repeats. The modified Bac-ELP1*-H1 had a T_t 1–2 °C higher than Pen-ELP1-H1 at each concentration, but the T_t was still in the range for use in mild hyperthermia experiments (Fig. 2E). The optimal treatment range for Bac-ELP1*-H1 was estimated to be from 15.2 μM to 64 μM (Fig. 2F).

3.3. Cellular uptake/association of the CPP-ELP-H1 polypeptides

In order to test the ability of CPPs to mediate cellular association and/or uptake of the ELP-H1 polypeptide, CPP-ELP-H1 polypeptides were labeled with fluorescein. MCF-7 cells were treated with the

Fig. 2. Temperature-induced aggregation of CPP-ELP-H1 polypeptides. The aggregation profile of the Pen-ELP1-H1 (A), Tat-ELPa80-H1 (C), and Bac-ELP1*-H1 (E) polypeptides was determined by monitoring the turbidity of solutions of various concentrations of each construct while heating at 1 °C/min. Absorbance data was converted to percentage of the maximum absorbance in order to view all curves on the same plot. The T_t was defined as the temperature at which the absorbance reaches 50% of the maximum. The T_t of each polypeptide was plotted versus polypeptide concentration (B, D, and F) and fitted with a logarithmic equation. The fit was used to determine the concentrations at which the T_t was 37 °C and 42 °C for each polypeptide (denoted by the dashed lines in the plots).

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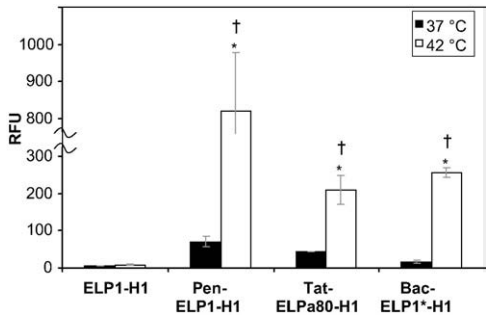

Fig. 3. Cellular uptake/association of the CPP-ELP-H1 polypeptides. MCF-7 cells were treated for 1 h at 37 or 42 °C with fluorescein labeled polypeptides. Levels of each polypeptide were assessed using flow cytometry (n=5000 cells). Forward and side scatter gating were used to eliminate cell debris from the analysis, and fluorescence data was normalized to cellular autofluorescence and corrected for variations in labeling efficiency among the polypeptides. Data represents the average of 3 experiments; error bars, SEM. *Difference between 37 and 42 °C levels is statistically significant (ANOVA, $p<0.01$). †Difference is significant as compared to ELP at 42 °C. (ANOVA, $p<0.01$).

labeled polypeptides for 1 h at 37 or 42 °C. Immediately after the treatment, the cells were harvested with a non-enzymatic cell dissociation buffer to avoid degradation of polypeptide on the cell surface, and the mean cellular fluorescence intensity was determined by flow cytometry. This assay ascertains the total amount of polypeptide taken up by the cells and bound to the outer surface of the plasma membrane. Previous studies have determined that CPP-ELP polypeptides are initially bound to the cell surface and are slowly internalized over 24 h [14,17]. When treated at 37 °C, Pen was the most efficient peptide for enhancement of cellular association/uptake, with Tat and Bac delivering 1.6 and 4.4 fold less polypeptide, respectively (Fig. 3). However, these results did not reach statistical significance. When aggregation was induced with hyperthermia treatment, the cellular association/uptake of Pen-ELP1-H1 was increased 13 fold, the association/uptake of Tat-ELPa80-H1 was increased 5 fold, and the Bac-ELP1*-H1 association/uptake was increased 16 fold as compared to each polypeptide at 37 °C ($p<0.01$). However, the total level of polypeptide bound to/taken up by the cells under hyperthermia treatment was greatest for Pen-ELP1-H1, with Bac-ELP-H1 accumulating 3.6 fold less and Tat-ELPa80-H1 accumulating 4.4 fold less ($p<0.01$). In summary, under hyperthermia conditions, all cell penetrating peptides tested significantly increased cellular association/uptake of the ELP-based polypeptides as compared to ELP-H1 lacking a CPP, and the penetratin peptide was the most efficient for the enhancement of association/uptake of ELP aggregates, followed by Bac then Tat.

3.4. Inhibition of cell proliferation by the CPP-ELP-H1 polypeptides

All CPP-ELP-H1 constructs were tested for their ability to inhibit the proliferation of MCF-7 cells. Cells were treated for 1 h at 37 or 42 °C with various concentrations of Pen-ELP1-H1, Tat-ELPa80-H1, and Bac-ELP1*-H1, and the cell number was determined 7 days later using the MTS assay. Also, each CPP-ELP control, lacking the H1 peptide, was tested in order to determine if any of the CPP-ELP constructs was toxic to MCF-7 cells. Pen-ELP1-H1 showed little effect on cell proliferation (Fig. 4A and B). Tat-ELPa80-H1 did inhibit the proliferation after 7 days, and its effect was enhanced when hyperthermia was applied during treatment (Fig. 4D). However, Tat-ELPa80, which lacks the H1 peptide, also showed significant toxicity, and all of the inhibition seen can be contributed to this toxicity (Fig. 4C). Bac-ELP1*-H1 was the most potent inhibitor of cell proliferation, and its effect was enhanced greatly by hyperthermia treatment (Fig. 4F). The Bac-ELP1 control did have some toxicity (Fig. 4E), but not as much as the Bac-ELP1*-H1 construct. For comparison of CPP toxicity, the data from each CPP-ELP after 42 °C treatment was overlaid (Fig. 4G). Both Tat-ELP and Bac-ELP showed some toxicity following hyperthermia treatment. Also, for comparison of the c-Myc inhibiting polypeptides, the data from each CPP-ELP1-H1 after 42 °C treatment was overlaid (Fig. 4H). It is clear that Bac-ELP1*-H1 is by far the most potent of the constructs tested, and its inhibition cannot be fully explained by CPP toxicity. It is interesting that Bac-ELP-H1 is much more potent than Pen-ELP-H1 in spite of its lower cellular uptake. In order to further investigate the reason for the enhanced antiproliferative effect of Bac-ELP-H1, the subcellular localization of the polypeptides was assessed.

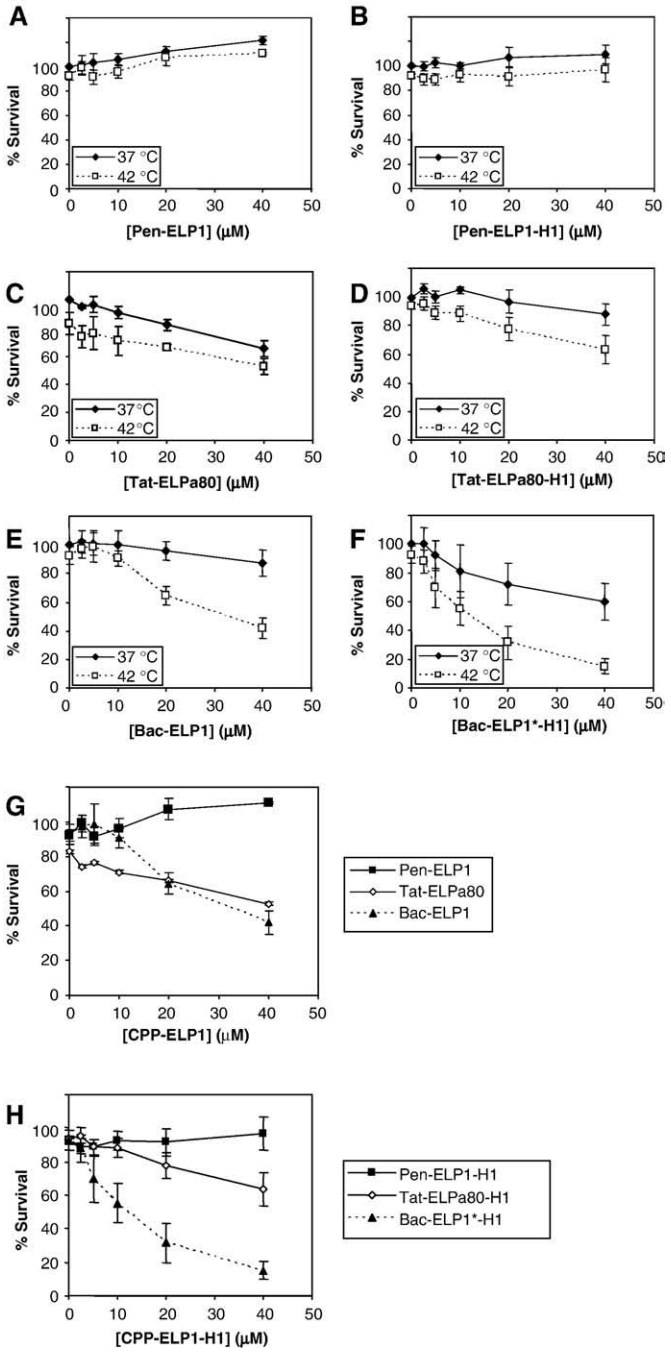

Fig. 4. MCF-7 proliferation after CPP-ELP-H1 treatment. MCF-7 cells were treated for 1 h at 37 or 42 °C with various concentrations of Pen-ELP1-H1 (B), Tat-ELPa80-H1 (D), or Bac-ELP1*-H1 (F), or the respective control polypeptides lacking the H1 sequence (A, C, and E), and the cell viability was determined after 7 days using the MTS assay. G. Overlay of the 42 °C data for each CPP-ELP. H. Overlay of the 42 °C data for each CPP-ELP-H1.

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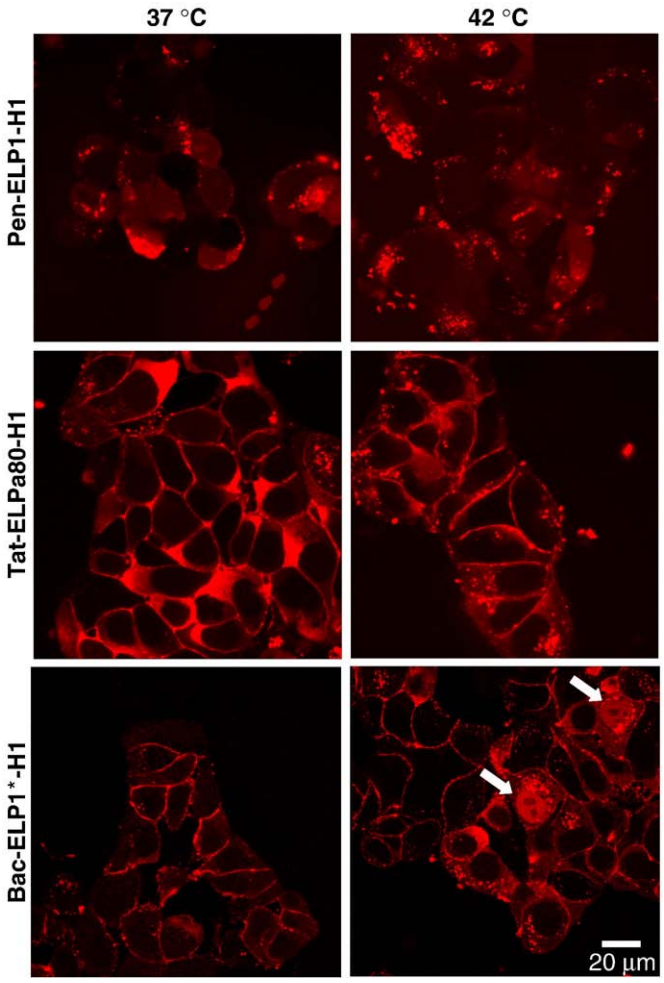

Fig. 5. Subcellular localization of CPP-ELP-H1 polypeptides. MCF-7 cells were treated for 1 h at 37 or 42 °C with rhodamine labeled polypeptides. Cells were imaged 24 h after treatment with a laser scanning confocal microscope. Image intensities were adjusted for clarity and do not represent the actual level of polypeptide in the cells.

3.5. Intracellular localization of the CPP-ELP-H1 polypeptides

To visualize the intracellular localization of the CPP-ELP-H1 polypeptides, MCF-7 cells were treated with rhodamine-labeled polypeptides for 1 h at 37 or 42 °C. The subcellular distribution was determined after 24 h by confocal fluorescence microscopy. As described previously [14], Pen-ELP1-H1 displayed a punctate cytoplasmic distribution at 37 °C, and large aggregates of Pen-ELP1-H1 could be seen in the cytoplasm following 42 °C treatment (Fig. 5, top panel). The distribution of Tat-ELPa80-H1 was similar to Pen-ELP1-H1, with cytoplasmic staining and aggregates present after hyperthermia treatment (Fig. 5, middle panel). Bac-ELP1*-H1, in contrast, showed intense plasma membrane staining at 37 °C, with a small amount of polypeptide present in the cytoplasm (Fig. 5, lower left). After hyperthermia treatment, Bac-ELP1*-H1 displayed a more diffuse distribution with some polypeptide and polypeptide aggregates in the cytoplasm. Also, in a subset of cells, Bac-ELP1*-H1 showed very bright nuclear staining exclusive of nucleoli (Fig. 5, lower right, arrows).

3.6. Time and concentration of dependence of Bac-ELP-H1 nuclear localization

In order to determine whether Bac-ELP1*-H1 nuclear localization is dependent on concentration and time, MCF-7 cells were treated with the polypeptide at concentrations ranging from 5 to 40 μM for 1 h, and the localization 24 h after treatment was observed. The percentage of cells containing nuclear localization was scored after staining the nuclei with Hoescht 33342 by counting 100 cells per sample using an epifluorescence microscope. High quality images of cells were obtained after staining the nuclei with Sytox green by confocal fluorescence microscopy. As shown in Fig. 6A, Bac-ELP1*-H1 showed plasma membrane and diffuse cytoplasmic localization after treatment at 37 °C, with little polypeptide present in the nucleus (left

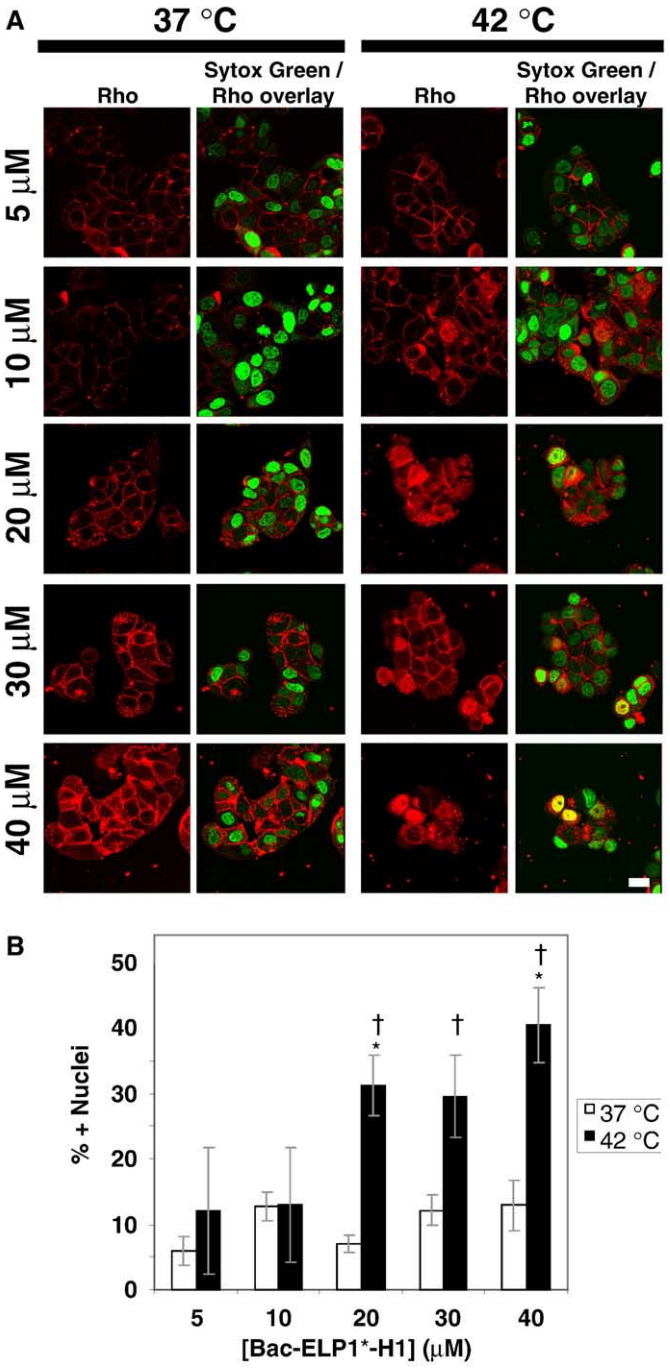

Fig. 6. Concentration dependence of Bac-ELP1*-H1 nuclear localization. A. MCF-7 cells were treated for 1 h at 37 or 42 °C with the indicated concentration of Bac-ELP1*-H1. Cells were fixed, stained with Sytox green, and imaged 24 h after treatment with a laser scanning confocal microscope. A representative image from each treatment condition is shown. (Sytox green images not shown) B. Cells were treated as in A, but were stained with Hoescht 33342 and imaged using an epifluorescence microscope. One hundred cells were observed per sample and scored for nuclear localization of the polypeptide. Results represent the mean ± SE of 3 independent experiments. *Difference between 37 and 42 °C levels is statistically significant (ANOVA, $p<0.01$). †Difference is significant as compared to ELP at 37 °C. (ANOVA, $p<0.01$).

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panel). Only about 10% of cells observed had nuclear polypeptide at all concentrations tested (Fig. 6B). However, 24 h after a 42 °C polypeptide treatment, a significant number of cells contained strong nuclear fluorescence (Fig. 6A, right panel). The percentage of cells with nuclear Bac-ELP1*-H1 increased with polypeptide concentration and with induction of polypeptide aggregation by hyperthermia (Fig. 6B, $p<0.01$). At the highest polypeptide concentration tested, about 40% of cells contained Bac-ELP1*-H1 in the nucleus. The nuclear localization of Bac-ELP*-H1 was not an artifact of cell fixation, as a similar distribution was observed in live cells (data not shown).

The time dependence of Bac-ELP1*-H1 nuclear localization was also determined using this assay. MCF-7 cells were treated with 30 μM Bac-ELP1*-H1 for 1 h at 42 °C, and the cells were observed 4, 24, 48, and 72 h after treatment. Fig. 7A shows that 4 h after treatment, the majority of the polypeptide is localized in the plasma membrane and cytoplasm, and nuclear localization is only rarely observed. However, by the 24 h time point, a large number of cells contain Bac-ELP1*-H1 in the nucleus. When cells were counted, the percentage containing nuclear polypeptide was only about 6% 4 h after treatment, but increased to 20 to 30% at time points from 24 to 72 h later (Fig. 7B). However, this trend was not statistically significant. This data demonstrates that Bac can deliver the ELP-H1 polypeptide into the cell nucleus, and nuclear localization is concentration dependent and is enhanced by hyperthermia treatment.

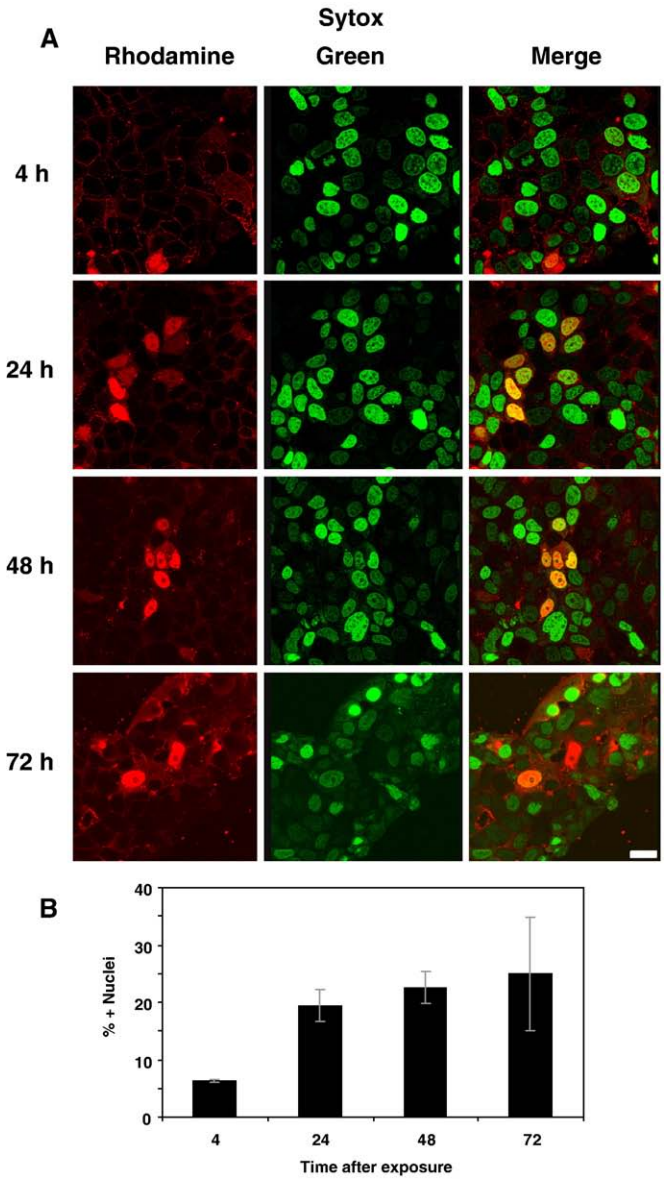

Fig. 7. Time dependence of Bac-ELP1*-H1 nuclear localization. A. MCF-7 cells were treated for 1 h at 37 or 42 °C with 30 μM Bac-ELP1*-H1. Cells were fixed at the indicated time after treatment, stained with Sytox green, and imaged with a laser scanning confocal microscope. A representative image from each treatment condition is shown. B. Cells were treated as in A, but were stained with Hoescht 33342 and imaged using an epifluorescence microscope. One hundred cells were observed per sample and scored for nuclear localization of the polypeptide. Results represent the mean±SE of 3 independent experiments.

4. Discussion

In order to enhance the potency of a thermally responsive c-Myc inhibitory polypeptide, three CPPs were evaluated for their ability to enhance the cellular binding and/or uptake and target the intracellular distribution of the polypeptide. The Pen peptide caused the greatest enhancement of ELP-H1 cellular uptake/association. The Tat and Bac peptides both enhanced the ELP-H1 uptake/association by MCF-7 cells, but not to the levels of Pen. This study also provides further evidence for the Bac peptide's ability to act as a CPP for a large protein. It was previously shown to allow cellular entry of avidin [26], and this study demonstrates that it may be universally applied for intracellular delivery of macromolecules.

The cellular uptake/association of all CPP-ELP polypeptides was significantly enhanced by hyperthermia treatment. Hyperthermia causes aggregation of the ELP-based polypeptides in the cell culture media, and the CPP mediates attachment of the polypeptide aggregates to the cell membrane. Previous studies with Pen-ELP and Tat-ELP used confocal microscopy and flow cytometry to demonstrate that these polypeptide aggregates were initially present on the outer surface of the cell membrane, and were slowly internalized by an endocytic mechanism [14,17]. Polypeptide aggregates were observed in the cell cytoplasm, confirming entry of the large aggregates into the cell. Therefore, during hyperthermia, the role of the CPP is to mediate attachment of these aggregates to the cell surface, where they are poised for endocytic cell entry.

When the intracellular localization of the CPP-ELP-H1 polypeptides was examined, Pen-ELP-H1 and Tat-ELP-H1 were localized to the cytoplasm, and Bac-ELP-H1 localized to both the cytoplasm and the nucleus. The Pen-ELP and Tat-ELP localization was consistent with previous studies. Pen-ELP and Tat-ELP were found to localize to the cytoplasm after endocytotic entry [17], and the Pen-ELP and Tat-ELP polypeptides were shown to deliver the H1 peptide [14] and doxorubicin [15], respectively, to the cytoplasm. In contrast to Pen and Tat, the Bac peptide was able to deliver the ELP-H1 polypeptide to the nucleus in a percentage of cells. The nuclear localization was very bright relative to the amount of polypeptide in the cytoplasm, and it appeared to be nucleoplasmic and excluded from nucleoli. The fraction of cells containing nuclear Bac-ELP-H1 increased with hyperthermia treatment and with polypeptide concentration.

The Bac peptide was previously shown to traffic into the nucleus [26]. This study extended the previous work to demonstrate that Bac can actively target a large polypeptide into the nucleus. Our hypothesis is that Bac is actively shuttling the polypeptides into the nucleus in a mechanism similar to a nuclear localization sequence (NLS). The Bac peptide is rich in arginines and prolines, a feature that distinguishes it from the lysine rich Pen and Tat peptides. Though it does not match the current model for a putative NLS [47] exactly, we believe a NLS-like mechanism is the most likely explanation for the nuclear localization of Bac-ELP and not Tat-ELP or Pen-ELP. Enhanced nuclear localization was seen in the samples that had been treated with Bac-ELP in combination with hyperthermia. This phenomenon is likely due to the fact that, when aggregation is induced, more polypeptide is delivered into the cell. Therefore, the enhanced nuclear localization is likely the result of a higher intracellular polypeptide concentration and not of direct nuclear uptake of polypeptide aggregates.

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Nuclear localization has been described previously for some Tat-delivered macromolecules. Tat has been used to deliver various types of cargo including small molecules, synthetic polymers, nanoparticles, liposomes, and nucleic acids; and these Tat fusions have been reported to localize to both the cytoplasm and the nucleus depending on the cargo and the cell type studied (reviewed in [48]). For example, a fusion of the Tat peptide and the chemotherapeutic doxorubicin (Dox) localized to the perinuclear region and the cytoplasm [49]. A fusion of the Tat peptide and the synthetic copolymer HPMA localized to both the cytoplasm and the nucleus [50,51]. Additionally, dextran coated SPIO nanoparticles fused with Tat localized to both the cytoplasm and the nucleus of lymphocytes [52]. Therefore, the subcellular localization of Tat-fused molecules is not predictable a priori as it is dependent on the cell type and the manner of cargo being delivered. It is clear from this study that the Tat peptide cannot effectively target ELP to the nucleus, but it should be noted that Tat-ELP may still be valuable for delivery of therapeutics with cytoplasmic targets.

Of the three CPPs tested, Bac-ELP-H1 was the most potent inhibitor of MCF-7 cell proliferation. Pen-ELP-H1 had no effect on proliferation in the 7 day assay used in this study. This is consistent with the previously published results that Pen-ELP-H1 acts by slowing the rate of proliferation of MCF-7 cells, and 11 days after treatment are needed to observe significant proliferation inhibition [14]. Tat-ELP-H1 did have some toxicity in MCF-7 cells after 7 days, but the control Tat-ELP polypeptide had similar toxicity, indicating that the effect was due to the Tat CPP. The toxicity of Tat-ELP has been observed previously [15] and was recently studied in detail. Tat-ELP induced membrane leakage and caspase-dependent apoptosis in MCF-7 cells when treatment was combined with hyperthermia [53]. This toxicity was unique to Tat-ELP1 in combination with hyperthermia, as no toxicity was observed upon Tat-ELP1 treatment at 37 °C or upon treatment with the Tat peptide or with ELP1 (lacking Tat). Also, some toxicity was observed with the Bac-ELP polypeptide when combined with hyperthermia treatment, and this likely occurs by a similar mechanism to the Tat-ELP toxicity. However, the toxicity from Bac-ELP was small in comparison to the effect observed with Bac-ELP-H1. The enhanced potency of Bac-ELP-H1 relative to the other polypeptides in this study is most likely due to its nuclear localization. We previously demonstrated that the Pen-ELP-H1 polypeptide was capable of preventing nuclear localization of c-Myc [14]. This resulted in the inability of newly translated c-Myc protein to interact with its dimerization partner Max. A portion of the Bac-ELP-H1 polypeptide localized to the cytoplasm, and this fraction may be responsible for some inhibition of cell proliferation via the c-Myc cytoplasmic sequestration mechanism previously observed. However, the enhanced potency of Bac-ELP-H1 relative to Pen-ELP-H1 and Tat-ELP-H1 is likely due to its ability to enter the nucleus and prevent c-Myc and Max dimerization or the c-Myc and INI1 interaction directly.

In recent years, CPPs have emerged as useful tools for intracellular delivery of many types of macromolecules. The work described here elucidates the fact that not only can CPPs enhance cellular uptake of polypeptides, they can direct their intracellular localization. This tool is especially useful when designing macromolecular polypeptide carriers for inhibitory peptides. When using a peptide with a cytoplasmic target, a CPP such as Pen or Tat can be added to the polypeptide carrier. When the goal is to deliver a therapeutic agent into the nucleus, the Bac CPP can be used. Given its potency and specificity for c-Myc, Bac-ELP-H1 is a leading candidate for further development as a thermally targeted cancer therapeutic. This polypeptide combines the advantages of thermal targeting to the tumor site with molecular targeting of c-Myc, making it an optimized therapeutic with dual target specificity.

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